UNITED STATES PATENT APPLICATION FOR:

RECOMBINANT MYCOBACTERIA OVEREXPRESSING D-ALANINE LIGASE GENE AND USES THEREFORE

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RECOMBINANT MYCOBACTERIA OVEREXPRESSING D-ALANINE LIGASE GENE AND USES THEREFORE

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001]   This application is related to U.S. Provisional Application Serial No. 60/434,200,

filed on December 17, 2002, which is incorporated herein by reference.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002]   The present invention was sponsored by the United States Department of

Agriculture Cooperative State Research Service Project under contract number NEB 14-108 and

the National Institute of Health under contract number RO3 A1051176-01. The government may

have certain rights in the invention.

BACKGROUND OF THE INVENTION

[0003]   The present invention relates to recombinant mycobacterial strains overexpressing

essential biosynthetic enzymes of pathogenic mycobacteria and to methods for using these

strains. More specifically, the present invention relates to recombinant mycobacteria strains

overexpressing D-alanine ligase (Ddl) and the use of such strains in *in vitro* methods for

identifying antimycobacterial agents directed against Ddl.

[0004]   The bacterial cell wall is an ideal target for drug design since similar structures

and biosynthetic pathways are absent from mammalian hosts. The lipid-rich mycobacterial cell

wall acts as an efficient permeability barrier (Brennan and Nikaido, 1995). Peptidoglycan, the

backbone of this structure, contains the D-amino acids D-alanine, D-glutamate, and

diaminopimelate, which may contribute to its stability against proteolytic degradation. D-

Alanine is one of the central molecules of the cross-linking step of peptidoglycan assembly.

Peptidoglycan biosynthesis in mycobacteria follows pathways similar to those in other eubacteria

(Belanger and Inamine, 2000). There are three enzymes involved in the D-alanine branch of

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peptidoglycan biosynthesis: the pyridoxal phosphate-dependent D-alanine racemase (Alr), the

ATP-dependent D-alanine:D-alanine ligase (Ddl), and the ATP-dependent D-alanine:D-alanine-

adding enzyme (MurF) (Walsh, 1989). D-Cycloserine (DCS; 4-amino-3-isoxazolidinone) is a

rigid analog of D-alanine and targets both Alr and Ddl in *Escherichia coli* (Lambert and

Neuhaus, 1992; Neuhaus, 1967). DCS also inhibits *Mycobacterium tuberculosis* Alr and Ddl

enzymes (David et al., 1969; Strych et al., 2001), suggesting that both Alr and Ddl are targets of

DCS in mycobacteria.

[0005]   DCS is effective against mycobacteria and is recommended to treat multidrug-

resistant *M. tuberculosis* in the DOTS-Plus management plan (Farmer, 2001; World Health

Organization, 2000). However, undesirable side effects restrict its use in human chemotherapy

(Yew et al., 1993). Nonetheless, the potent bactericidal effect of DCS against mycobacteria

makes this drug an attractive prototype compound to develop novel antimycobacterial agents. In

addition, identification of the lethal target(s) of DCS action would allow for the rational design

of new antimycobacterial drugs, structurally related or unrelated to DCS, targeting enzymes of

the D-alanine pathway of peptidoglycan biosynthesis. Moreover, these types of inhibitors may

weaken the cell wall and act synergistically with other antimicrobial agents (Rastogi et al.,

1990). In the early 1970s, David (1971) isolated and characterized step-wise DCS-resistant *M.*

tuberculosis mutants that showed either normal or reduced cellular permeability to DCS and

speculated that Alr plays only a minor role in the mechanism of action of DCS. *Mycobacterium*

smegmatis, a nonpathogenic species, is a useful model to study drug resistance mechanisms in

pathogenic mycobacteria, especially when conserved cellular processes are involved (Reyrat and

Kahn, 2001; Tyagi and Sharma, 2002). Peteroy et al. (2000) described the isolation and

characterization of an *M. smegmatis* mutant resistant to both DCS and vancomycin though the

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molecular basis of the resistance mechanism remains unknown. In previous studies, we identified a spontaneous DCS-resistant *M. smegmatis* mutant with a promoter-up mutation in the *alr* gene, resulting in the overproduction of the Alr enzyme (Caceres et al., 1997). Alr was shown to be inhibited by DCS in a concentration-dependent manner, and DCS resistance could be conferred to pathogenic mycobacteria carrying the *M. smegmatis alr* gene in a multicopy plasmid. In addition, DCS was shown to competitively inhibit the native Ddl enzyme from *M. tuberculosis* (David et al., 1969). Belanger et al. (2000) reported the characterization of a temperature-sensitive *M. smegmatis* mutant with a single amino acid substitution in Ddl. The mutant was more susceptible to DCS, and the temperature sensitivity phenotype was due to the decreased activity of the mutated enzyme.

[0006] Recently, we observed that *M. smegmatis alr* null mutants are not dependent on D-alanine for growth, suggesting the existence of another pathway for D-alanine biosynthesis (Chacon et al., 2002). In addition, the *alr* null mutant is hypersusceptible to DCS, suggesting that a lethal target other than Alr is responsible for the bactericidal effect of DCS.

[0007] In order to develop novel antimicrobial agents structurally related to DCS, the lethal target(s) of DCS need to be identified and methods of screening for inhibition need to be developed.

BRIEF SUMMARY OF THE INVENTION

[0008] In this study, we investigated the roles of both Alr and Ddl in the mechanisms of action of and resistance to DCS in *M. smegmatis*. We demonstrate that Ddl activity is inhibited by DCS in a concentration-dependent manner. Overexpression of the *ddl* gene confers resistance to DCS but not to ß-chloro-D-alanine (ßCDA), an inhibitor of D-alanine racemase. Furthermore, a strain overexpressing both the *alr* and *ddl* genes displayed an increased level of resistance to DCS. Analysis of the intracellular alanine pools in wild-type and recombinant *M. smegmatis*

strains demonstrated that Ddl activity is not significantly affected by DCS at concentrations inhibiting Alr.

[0009]   The present invention is directed to recombinant mycobacterial strains which overproduce D-alanine ligase (Ddl), to cloned isolated genes encoding Ddl, and to the uses of the recombinant strains, and heterologous proteins.

[0010]   In one aspect of the present invention, recombinant *M. smegmatis* strains expressing heterologous or overexpressing native Ddl are provided. In one embodiment of this aspect, the Ddl is the product of *M. smegmatis ddl* gene. In another embodiment, the Ddl is the product of *M. tuberculosis ddl* gene. In yet another embodiment, the Ddl is the product of both *M. smegmatis* and *M tuberculosis ddl* genes.

[0011]   In another aspect of the invention, purified recombinant Ddl is provided.

[0012]   In a further aspect of the invention, cell-free methods for screening for antimicrobial agents which target the D-alanine ligase, an enzyme in the D-alanine branch of mycobacterial peptidoglycan synthesis are provided. In one embodiment of this aspect, the antimicrobial agent is structurally related to D-cycloserine. In another embodiment, the antimicrobial agent is unrelated to DCS. In a further embodiment of this aspect, the screening method employs a thin-layer chromotography assay to detect Ddl enzyme activity.   In yet another embodiment, the screening method employs a phosphate release assay.

[0013]   In another aspect of the invention, *in vitro* methods for detecting bactericidal activity of compounds against pathogenic mycobacteria are provided. The methods comprise the use of overexpressing recombinant mycobacterial strains In one embodiment of this aspect, the recombinant mycobacterial strain is GPM259. In another embodiment, the recombinant strain is GPM260. In a further embodiment, the recombinant strain is GPM265.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] FIG 1 shows primer extension analysis of the *M. smegmatis* and *M. tuberculosis* *ddl* transcripts. Total RNA (40 µg) from mc^{2}155, GPM259, and GPM265 was annealed with primer SMDDLPE (A) or TBDDLPE (B) and extended as described in Materials and Methods. Lanes A, C, G, and T display a dideoxy sequencing ladder with the corresponding primer. Asterisks, transcriptional start sites. The putative – 10 boxes are highlighted. The start codon (GTG) for the *M. smegmatis ddl* gene is boxed.

[0015] FIG 2 shows autoradiograms of the TLC for typical Ddl assays. Ddl activities in crude extracts were determined as described in Materials and Methods. (A) Linear increase in the synthesis of the D-alanine:D-alanine dipeptide at increasing amounts of GPM259 cell extract. The percentage of conversion was defined as the percentage of the total radioactivity present in the [^{14}C]D-Ala: D-Ala dipeptide spot. (B) Percent inhibition of dipeptide formation (percent inhibition = 100 – 100 × [percent conversion$_{\text{with DCS}}$/percent conversion$_{\text{without DCS}}$]) in GPM259 cell extract (8 µg of total protein) at increasing concentrations of DCS.

[0016] FIG 3 shows analysis of Dir and Alr activities in *M. smegmatis* strains. Specific activities for Ddl (A) and Alr (B) in crude extracts were determined as described in Materials and Methods. Specific activities are expressed as micromoles of substrate (L-alanine for Alr enzyme assay and D-alanine for Ddl enzyme assay) per milligram per minute (means ± standard deviations of triplicate measurements).

[0017] FIG 4 shows inhibitory effect of DCS on *M. smegmatis* Ddl activities. Enzyme activities were determined in cell crude extracts from mc^{2}155 (solid squares), GPM14 (solid triangles), GPM259 (open triangles), GPM260 (open squares), and GPM265 (solid diamonds) in the presence of increasing concentrations of DCS. Percent inhibition was calculated (percent

inhibition = 100 − 100 [$SA_{with\ DCS}/SA_{without\ DCS}$]; SA, specific activity) from three independent experiments. Statistical analysis was performed as described in Materials and Methods. The inhibition patterns can be divided into two groups (group I, mc^2155, GPM14, GPM259, and GPM260; group II, GPM265). A significant difference between groups was detected (P = 0.02), while no significant differences within group I were detected (P = 0.28).

[0018]   FIG 5 shows inhibition of colony formation by DCS for various *M. smegmatis* strains. The curves were generated from data of a representative experiment with mc^2155 (solid squares), GPM2 (open squares), GPM259 (open triangles), GPM265 (solid triangles), GPM14 (solid diamonds), and GPM260 (open diamonds). Statistical analysis for three independent experiments was performed as described in Materials and Methods. Susceptibilities to DCS can be divided into four groups (group I, mc^2155 and GPM2; group II, GPM259 and GPM265; group III, GPM14; group IV, GPM260). Significant differences between groups were detected (group I versus group II, P < 0.003; group II versus group III, P = 0.025; group III versus group IV, P = 0.020), while no significant differences within groups were detected (P ≥ 0.881).

[0019]   FIG 6 shows characteristic HPLC chromatographic profiles of *M. smegmatis* intracellular amino acid pools. Intracellular amino acid pools of *M. smegmatis* mc^2155 untreated (A) and treated with DCS (B) were prepared and analyzed by a reverse-phase HPLC method as described in Materials and Methods. The synthetic amino acid L-*homo*-arginine (L-hArg) served as an internal standard for quantifying the abundance of each individual amino acid Peaks for various amino acids are indicated, with L- and D-alanine highlighted.

[0020]   FIG 7 shows intracellular pool of alanine in *M. smegmatis* strains Exponentially *growing M.f smegmatis* cells in minimal media were split into two subcultures, and DCS was added to a final concentration of 75 µg ml^{-1} to one of the subcultures Cells were harvested after

2 h of incubation, and intracellular amino acid pools were determined as described in Materials

and Methods The intracellular pool of alanine (full-length bars) is expressed relative to the L-

glutamate pool ([Ala]/ L-Glu]). The relative abundance of each stereoisomer in the pool is also

shown by open (L-alanine) and solid (D-alanine) bars and indicated in the table at the bottom.

Values shown are means ± standard deviations of three independent experiments. Analysis of

variance was used to compare values for total alanine, L-alanine, and D-alanine pools in each

strain for both DCS-treated and untreated cells. Significant differences were observed for the L-

alanine pools (P = 0.03). For D-alanine pools, significant differences (asterisks) were observed

only for strains GPM259 (P < 0.05), GPM14 (P < 0.01), and GPM260 (P < 0.01).

DETAILED DESCRIPTION OF PREFERRED EMBODIMENT

[0021]   D-Cycloserine (DCS) targets the peptidoglycan biosynthetic enzymes D-alanine

racemase (Alr) and D-alanine:D-alanine ligase (Ddl). Previously, we demonstrated that the

overproduction of Alr in *Mycobacterium smegmatis* determines a DCS resistance phenotype. As

described in further detail herein, we investigated the roles of both Alr and Ddl in the

mechanisms of action of and resistance to DCS in *M. smegmatis*. It was discovered that the

overexpression of either the *M. smegmatis* or the *Mycobacterium tuberculosis ddl* gene in *M.*

smegmatis confers resistance to DCS, but at lower levels than the overexpression of the *alr* gene.

Furthermore, a strain overexpressing both the *alr* and *ddl* genes displayed an eightfold-higher

level of resistance. The alanine pools in *M. smegmatis* wild-type and recombinant strains with or

without DCS treatment were determined. Alr-overproducing strain GPM14 cells not exposed to

DCS displayed almost equimolar amounts of L- and D-alanine in the steady state. The wild-type

strain and Ddl-overproducing strains contained a twofold excess of L- over D-alanine. In all

strains, DCS treatment led to a significant accumulation of L-alanine and a concomitant decease

of D-alanine, with approximately a 20-fold excess of L-alanine in the Ddl-overproducing strains.

These data suggest that Ddl is not significantly inhibited by DCS at concentrations that inhibit Alr. The present invention is based on the above discoveries which suggest that Ddl is the lethal target of DCS and thus allow for identification of novel drugs targeting the D-alanine branch of mycobacterial peptidoglycan biosynthesis.

[0022]   The publications and other materials used herein to illuminate the background of the invention, and in particular cases, to provide additional details respecting the practice, are incorporated by reference and for convenience are referenced in the following text by author and date and are listed alphabetically by author in the appended List of References.

DEFINITIONS

[0023]   The present invention employs the following definitions:

[0024]   "alrA" and "alr" refer to the D-alanine racemase gene, including normal alleles of the alrA gene.

[0025]   "Alr" and "Alr" refer to D-alanine racemase enzyme.

[0026]   "Displays increased susceptibility to antimycobacterial agent" refers to a reduction in the minimal inhibitory concentration of the mutant strain when compared with the wild type strain.

[0027]   "DCS" and "D-cycloserine" refer to 4-amino-3-isoxayolidinone.

[0028]   "ddl" refers to the D-alanine ligase gene including normal alleles of the ddl gene.

[0029]   "Ddl" refers to D-alanine-D-alanine synthetase, which is also referred to as D-alanine ligase.

[0030]   "Isolated polypeptide" refers to a polypeptide produced as an expression product of an isolated and manipulated genetic sequence, even if expressed in a homologous cell type.

Synthetically made forms or molecules expressed by heterologous cells are inherently isolated molecules.

[0031]   In previous studies, a genomic library from a DCS-resistant mutant constructed in a multicopy plasmid was introduced into the wild-type *M. smegmatis* strain and clones resistant to 300 μg of DCS ml⁻¹ were selected and isolated (Caceres et al., 1997). Using this strategy, we did not identify a recombinant clone carrying the *ddl* gene. Since target overproduction determines a drug resistance phenotype, this outcome was unexpected. To exclude any possible bias in this library, we also screened a *M. smegmatis* cosmid library in a similar manner. Surprisingly, a DCS resistant clone carrying the *ddl* gene was still not identified. Lowering the DCS concentration resulted in a high background of DCS-sensitive colonies. These data suggest that either the overexpression of the *ddl* gene is toxic to the host or that the level of overexpression is not sufficient to confer a selectable resistance phenotype under the selection conditions described. To test these hypotheses, the *M. smegmatis* and *M. tuberculosis ddl* genes were amplified from genomic DNA and cloned into the *E. coli-Mycobacterium* shuttle vector pMV262, carrying a kanamycin resistance marker. Recombinant plasmids were introduced into *M. smegmatis*, and kanamycin-resistant transformants were isolated.

[0032]   In this study, we demonstrated that Ddl enzyme activities in crude extracts were inhibited by DCS in a concentration-dependent manner, similar to the effect observed for the Alr enzyme (Caceres et al., 1997). Approximately 50% of Ddl enzyme activity is inhibited by DCS at a concentration of 200 μg ml, while only 10 to 15% of the Ddl activity is inhibited at 50 μg ml, near the MIC. The apparent discrepancy reflects the difference between *in vivo* and *in vitro* conditions. In live bacilli, DCS also inhibits Alr, resulting in a limited supply of D-Ala, which is

the substrate for Ddl, while in the cell-free assay, D-Ala is provided in excess. These data

confirmed that the mycobacterial Ddl enzyme is a target of DCS.

[0033] Overexpression of either the *M. smegmatis* or *M. tuberculosis ddl* gene, using the

same expression vectors as those used to overexpress Alr (Caceres et al., 1997), confers an

intermediate level of resistance to DCS. Furthermore, GPM260, a recombinant strain of *M.*

smegmatis overproducing both Alr and Ddl, was constructed and characterized. GPM260

displayed a higher level of resistance to DCS than its parent strain, GPM14, which is consistent

with the increased DCS resistance levels of *S. gordonii* mutants with elevated Alr and Ddl

activities (Reitz et al., 1967). To test the hypothesis that inhibition of Alr by DCS decreases the

intracellular pool of D-alanine, we compared the levels of D-alanine pools in wild-type and

recombinant *M. smegmatis* strains with or without DCS treatment. The results showed that DCS

treatment decreased the intracellular D-alanine pools in strains with wild-type Alr activity while

the Alr-overproducing strain GPM14 maintained a relatively abundant pool of D-alanine. This

observation is consistent with the intermediate level of resistance to DCS of Ddl-overproducing

strains. In addition, analysis of the alanine pools in Alr-Ddl-overproducing strain GPM260

suggests that, upon exposure to DCS, Alr is readily inhibited. In contrast, overproduction of Alr

and/or Ddl does not have an effect on the susceptibility to vancomycin. The increased resistance

of a previously isolated *M. smegmatis* mutant to both DCS and vancomycin (Peteroy et al., 2000)

may underscore a resistance mechanism different from overproduction of Alr and/or Ddl.

Regarding resistance to DCS, there has been only one fully characterized mechanism in

mycobacteria involving overexpression of the *alr* gene (Caceres et al., 1997). In this study, we

have shown that overexpression of the *ddl* gene also confers DCS resistance *in M. smegmatis,*

but at levels of resistance below those for strains overproducing Alr. Therefore, we do not

expect that mutational changes, for example, *ddl* promoter-up mutations, would play an important role in the emergence of naturally DCS-resistant strains. The presence of a mutation(s) in the *ddl* structural gene leading to DCS resistance has not been demonstrated and needs further investigation.

[0034] DCS targets both Alr and Ddl enzymes, but the lethal target for its bactericidal effect has not been identified *M. smegmatis* alr null mutants are not dependent on D-alanine for growth, indicating that there is another pathway for D-alanine biosynthesis (Chacon et al., 2002). In addition, the *M. smegmatis* alr mutant is hypersusceptible to DCS, underlying the existence of another lethal target, most likely Ddl. The DCS hypersensitivity phenotype of the *alr* mutant is also consistent with the essentiality of the *M. smegmatis ddl* gene, as suggested by the temperature sensitivity phenotype of a mutant carrying a single amino acid substitution in Ddl (Belanger et al., 2000) This temperature-sensitive mutant is also more susceptible to DCS, presumably due to the low activity of the mutated Ddl. Therefore, Ddl seems to be the lethal target of DCS, while the inhibition of Alr by DCS further decreases the D-alanine pool and contributes to the inhibition of Ddl. Previously, it was suggested that DCS resistance in *M. tuberculosis* is primarily due to mutations in the *ddl gene* (David et al., 1969), and it was further speculated that inhibition of Alr plays only a minor role in the mechanism of DCS action (David, 1971) Contrary to this, our data showed that Ddl is not significantly affected by DCS at the concentration that inhibits Alr. Our observation suggests that Alr overproduction contributes to the maintenance of the internal D-alanine pool, thus antagonizing the inhibition of Ddl by DCS. For the *Streptococcus faecalis* enzymes, the DCS inhibition constants for both Alr ($K_I = 0.02$ mM) and Ddl ($K_I = 0.9$ mM) have been determined from the purified enzymes (Neuhaus, 1967). If the K_Is for the mycobacterial enzymes were to follow a similar trend, an attractive hypothesis

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would be that the overproduction of a high-affinity DCS-binding target (Alr) would protect a

more fundamental and low-affinity target, probably Ddl, from drug inhibition. In this view, the

major mechanism of resistance is the overproduction of the high-affinity but dispensable target,

while the bactericidal effect is due to the inhibition of the low-affinity lethal target. Our studies

do not rule out the possibility that the bactericidal action of DCS may result from the inhibition

of a lethal target(s) other than Ddl. Further research is necessary to assess the lethal targets in

the D-alanine branch of peptidoglycan biosynthesis in mycobacteria. This information is

necessary for the development of new antimycobacterial agents targeting the D-alanine pathway.

[0035] The practice of the present invention employs, unless otherwise indicated,

conventional techniques of chemistry, molecular biology, microbiology, recombinant DNA and

genetics. See, e.g., Maniatis et al. (1982); Sambrook et al. (1989); Ausubel et al. (1992); Guthrie

and Fink (1991); Weissbach and Weissbach (1986); Zaitlin et al. (1985) and Gelvin et al.

(1990).

[0036] Method of Use: Drug Susceptibility Assays

[0037] The bactericidal activity of lead compounds are evaluated *in vitro*. Conventional

protocols for determination of drug minimum inhibitory concentration (MIC) may be used.

Example 1D. employs one example of such assays. For example, the mycobacterial cells taken

from glycerol stock, or preferably from a single colony, are grown in either M-ADC-TW (for

faster growing strains such as *M. smegmatis*) or MO-ADC-TW (for slow growing strains such as

M. avium or *M. bovis* BCG). Growth is continued in the absence of test compound until the

optical density of 600 nm reaches approximately 0.1 to 1.0. Medium is supplemented with

nutrients, if necessary.

[0038] Approximately 10^5 CFU in 0.1 ml are inoculated in triplicate onto 96-well microplates containing either serial twofold dilutions of the test compound or without, for controls. Plates are incubated at 37° C and checked periodically for growth. MIC is determined as the lowest concentration at which there is no visible growth of bacteria. MIC is confirmed by consistent results from at least three independent cultures.

[0039] Alternatively, the bactericidal activity of the test compound can be evaluated by measuring D-lactate dehydrogenase activity present in the supernatant. D-lactate dehydrogenase is a bacterial cytosolic enzyme, and it is only released to medium after bacterial lysis. D-lactate dehydrogenase detection kits are commercially available and can be adapted easily to microplate format.

[0040] Method of Use: Inhibition Assays

[0041] The present invention is based on the evidence, reported in further detail herein: 1) that Ddl is a binding target of DCS and is inhibited in a concentration-dependent manner; 2) that Ddl is not significantly affected by DCS at the concentration that inhibits Alr; 3) that Alr overproduction contributes to the maintenance of the internal D-alanine pool, thus antagonizing the inhibition of Ddl by DCS; and 4) that a strain overexpressing alr and *ddl* genes displays increased level of resistance to DCS as compared to a strain overexpressing only the alr gene. These results, in view of previously reported results, indicate that Alr is a high-affinity but dispensable target of DCS, while Ddl is the low-affinity lethal target of the bactericidal action of DCS. Thus, according to the present invention, a method is provided for screening of antimycobacterial agents against Ddl by a variety of drug screening techniques.

[0042] Preferably, purified Ddl is obtained from *M. tuberculosis ddl* gene by PCR amplification and cloning into a proper expression shuttle plasmid vector, such as an *E. coli*

expression vector endowed with a mycobacterial replication origin and/or promoter, and

overexpressed in *M. smegmatis*. This host strain is modified by inactivation of its endogenous

ddl gene, so that it only expresses the *M. tuberculosis* counterpart. The *M. tuberculosis* Ddl

enzyme is purified as a polyhistidine-tagged fusion protein through a single-step affinity

chromatography in large amount and its identity is verified with biochemical methods. Ddl

catalyzes the reaction 2 D-alanine + ATP $\rightarrow$ D-alanyl-D-alanine + ADP + P*i* . Thus the purified

protein may be used in any of a variety of *in vitro* cell-free screen systems as are known in the

art.

[0043]   The thin-layer chromatography (TLC)-based D-alanine ligase assay described in

Example 1 can be used, however it is time consuming and cumbersome, and not readily

adaptable to microplate format suitable for high-throughput screen. A preferred assay is the

phosphate release assay (such as that used in PiPer Phosphate Assay Kit from Molecular Probes)

which offers high sensitivity and extraordinary adaptability. The establishment of such an assay

permits the screening of thousands of compounds, including, but not limited to, D-cycloserine

and modified or derivative D-cycloserine molecules, for potential inhibitors of D-alanine ligase.

[0044]   Promising candidate antimycobacterial agents are further tested *in vitro* using *M.*

smegmatis and *M. tuberculosis* reference strains (See Methods of Use: Drug Susceptibility

Assays). Preferably, the overproducing strains of the present invention are used at this step to

evaluate the potential bactericidal activity of the lead compounds compared with D-cycloserine.

In addition, the susceptibility of the reference strains to lead compounds indicate the specific

pathway or enzyme targeted.

[0045]   Method of Use: Analysis of Drug Action

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[0046]   According to the present invention a method is provided for analyzing the *in vivo* metabolic change in mycobacteria upon drug treatment. The free amino acid pool is indicative of the pathways which are either shut down or activated by the test drug. In addition, the alteration of free amino acid pool, especially the abundance of L- or D- stereotype of an individual amino acid, reflect the mode of action of the drug. This method is useful compared with cumbersome biochemical characterization of various bacterial components, such as cell wall analysis and lipid analysis. Previously, amino acid analysis has been used as a tool to determine the composition of purified protein or just simply quantify the amino acid level in biological fluids, however its use has not previously been reported for analysis of drug action.

[0047]   Method of Use: Vaccine Development

[0048]   The present invention is also useful for the development of a novel vaccine against pathogenic mycobacteria, such as, but not limited to, *M. tuberculosis, M. bovis, M. africanum, M. microti, M. leprae, M. avium, M. intracellular, M. paratuberculosis, M. ulcerans, M. marinum*, and subspecies and genetic variants thereof. A mycobacteria can be attenuated by selection of mutants that are better suited to growth in abnormal culture conditions and are therefore less capable of growth in the host. The attenuated mycobacterium may have inserted therein one or more DNA molecules for stimulation of an immune response directed against polypepticles encoded by the inserted nucleic acid molecule. Vaccines of the present invention may be formulated with conventional carriers and/or adjuvants.

[0049]   For obvious practical and moral reasons, initial work in humans to determine the efficacy o experimental compositions with regard to such afflictions is infeasible. Accordingly, in the early development of any drug or vaccine it is standard procedure to employ appropriate animal models for reasons of safety and expense. The success of implementing laboratory

animal models is predicted on the understanding that immunogenic epitopes are frequently active

in different host species. Thus, an immunogenic determinant in one species, for example a

rodent or guinea pig, will generally be immunoreactive in a different species such as in humans.

Only after the appropriate animal models are sufficiently developed will clinical trials in humans

be carried out to further demonstrate the safety and efficacy of a vaccine in man.

[0050]   With regard to alveolar or pulmonary infections by *M. tuberculosis*, the guinea

pig model closely resembles the human pathology of the disease in many respects. Accordingly,

it is well understood by those skilled in the art that it is appropriate to extrapolate the guinea pig

model of this disease to humans and other mammals. As with humans, guinea pigs are

susceptible to tubercular infection with low doses of the aerosolized human pathogen *M.*

tuberculosis. Unlike humans where the initial infection is usually controlled, guinea pigs

consistently develop disseminated disease upon exposure to the aerosolized pathogen, facilitating

subsequent analysis. Further, both guinea pigs and humans display cutaneous delayed-type

hypersensitivity reactions characterized by the development of a dense mononuclear cell

induration or rigid area at the skin test site. Finally, the characteristic tubercular lesions of

humans and guinea pigs exhibit similar morphology including the presence of Langhans giant

cells. As guinea pigs are more susceptible to initial infection and progression of the disease than

humans, any protection conferred in experiments using this animal model provides a strong

indication that the same protective immunity may be generated in man or other less susceptible

mammals. Those skilled in the art will appreciate that the present invention may be practiced

with various mammalian hosts including humans and domesticated animals.

[0051]   A strain of mycobacteria having a regulatable promoter to control the expression

of the *ddl* gene, whose product is responsible for the synthesis of D-alanyl-D-alanine, will

provide a attenuation of the mycobacterial strain. Use of a promoter that is active *in vitro* but is inactive, or minimally active, *in vivo* will cause lysis of the mycobacterial cells and release of immunogens which generate an immunological response. Replacement of the chromosomal wild type *ddl* gene with a regulatable *ddl* gene can be accomplished by means known in the art, including the use of a conventional integrating vector such as those described herein (See Example 1).

[0052]   The mycobacterial strain used may be a wild-type mycobacterial strain or, alternatively, an alr null mutant mycobacterial may be used, such as the alr mutant described in Appl'n Ser. No. U.S. 2003/0133952.

EXAMPLE 1

[0053]   The present invention is further described in the following examples, which are offered by way of illustration and are not intended to limit the invention in any manner. Standard techniques known in the art or the techniques specifically described below are utilized.

[0054]   Materials and Methods

[0055]   A. Bacterial strains, plasmids, and culture conditions

[0056]   Bacterial strains and plasmids used in this example are listed in Table 1. *E. coli* strains were grown at 37°C in Luria-Bertani broth or agar. *M. smegmatis* strains were grown at 37°C with shaking (200 rpm; Innova 4300 incubator shaker; New Brunswick Scientific, Edism, N.J.) in Middlebrook 7H9 broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with albumin-dextrose complex (ADC) and 0.05% Tween 80 (M-ADC-TW broth). For analysis of the intracellular amino acid pools, *M. smegmatis* was grown in a minimal medium based on the formulation of Zygmunt (1963) and modified as previously described (Chacon et al., 2002). Tryptic soy agar base (Difco Laboratories, Detroit, Mich.) was used for growth of *M. smegmatis*

on solid media. Transformations of *E. coli* and *M smegmatis* were performed by electroporation

with a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.) as described previously (Foley-

Thomas et al., 1995). The following antibiotics were added to the media when necessary:

kanamycin (Sigma Chemical Co., St. Louis, Mo.) at 50 µg ml^{-1} or carbenicillin (Sigma) at 50 µg

ml^{-1} for *E. coli* strains and kanamycin at 10 µg ml^{-1} for *M. smegmatis* strains.

[0057]   TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference and/or source
E. coli XL10- GOLD	Tet^r Δ*(mcrA)*183 Δ *(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F' *proAB lac^{q}ZΔM15* Tn*10* (Tet^r) Amy Cam^r]	Stratagene
M. smegmatis mc^{2}155	High-transformation *M. smegmatis* mutant derived from mc^{2}6	25
M. smegmatis GPM2	mc^{2}155(pMV262)	5
M. smegmatis GPM14	DCS-resistant mutant derived from mc^{2}155; overproducing Alr	5
M. smegmatis GPM259	mc^{2}155(pBUN250); overproducing Ddl	This work
M. smegmatis GPM260	GPM14(pBUN250); overproducing both Alr and Ddl	This work
M. smegmatis GPM265	mc^{2}155(pBUN276); overproducing *M. tuberculosis* Ddl	This work
M. smegmatis GPM198A	mc^{2}155(pBUN128A)	This work
pCR2.1	TA cloning vector	Invitrogen
pMV262	*E. coli-Mycobacterium* shuttle plasmid	MedImmune, Inc.; 7
pBUN128A	pMV262 with a 1.4-kb insert containing the complete *M. tuberculosis ddl* gene inserted at the *Eco*RI site	This work
pBUN172	Recombinant plasmid isolated from an *M. smegmatis* genomic library which hybridized with an internal fragment of the *ddl* gene	This work
pBUN250	pMV262 with a 1.4-kb insert containing the complete *M. smegmatis ddl* gene inserted at the *Eco*RI site	This work
pBUN276	pMV262 with the *M. tuberculosis ddl* gene inserted at	This works

the *BamHI/HindIII*
site; the *M. tuberculosis ddl* gene is fused with the
DNA sequence
corresponding to the first 6 codons of the *M. bovis*
BCG *hsp60* gene

[0058]   B. Oligonucleotide primers, nucleic acid manipulations, and primer extension

analysis.

[0059]   All oligonucleotide primers were from Integrated DNA Technologies, Inc.,

Coralville, Iowa. For PCR amplification of the complete *ddl* gene from *M. smegmatis* were

SMDDLCF (5'-CGC ATA AGG CCA GGT CAG-3'(SEQ ID NO:1) and SMDDLCR (5'-CGA

AAA ACC CGT CGT GC-3'(SEQ ID NO:2). The primers for PCR amplification of the *ddl* gene

from *M. tuberculosis* were DDLATBU (5'-GCT AAG TGC CGA TCG CAA G-3'(SEQ ID

NO:3) and DDLATBD (5'-ATA ACG CTG CTG CTG GGT C-3'(SEQ ID NO:4) and

TBDDLEXF (5'-CGG GAT CCG TGA GTG CTA ACG AC-3'(SEQ NO:5) and TBDDLEXR

(5'-CGG AAG CTT GTG CCG ATC GCA AGC-3'(SEQ ID NO:6). The primers SMDDLPE

(5'-AAA CGC TCC GGA TCG AGG TTG-3'(SEQ ID NO:7) and TBDDLPE (5'-GAG ATG

GCG TGC TCG TTG-3'(SEQ ID NO:8) were used in primer extension analysis for the *ddl*

mRNA of *M. smegmatis* and *M. tuberculosis*, respectively. PCR amplifications were performed

in a Perkin-Elmer GeneAmp 9600 thermal cycler (Roche Molecular Systems, Branchburg, N.J.)

by using the Expand high-fidelity PCR system (Roche Molecular Biochemicals, Indianapolis,

Ind.). For restriction digestions, ligations, and agarose gel electrophoresis, standard procedures

previously described (Sambrook et al., 1989) were followed. Total RNA from *M. smegmatis*

strains was isolated by using RNAWIZ (Ambion, Inc., Austin, Tex.) with minor modifications

(Bashyam and Tyagi, 1994). Primer extension analysis of the *ddl* mRNA was carried out as

described previously (Davis et al., 1994). The oligonucleotide was radiolabeled with [y-^{32}P]ATP

by using T4 polynucleotide kinase (Promega, Madison, Wis.), and the reactions were extended

with Moloney murine leukemia virus reverse transcriptase (Promega). Radioactivity in primer

extension products was quantified with a PhosphorImager by using ImageQuant, version 3.3

(Molecular Dynamics, Sunnyvale, Calif.).

[0060]   C. *M. smegmatis* genomic library construction and cloning of the *M. smegmatis*

ddl gene.

[0061]   Chromosomal DNA from *M. smegmatis* mc^{2}155 was prepared as described

previously (Whipple et al., 1987). For library construction, chromosomal DNA was partially

digested with *Sau*3AI, and fragments of 3.0 to 4.0 kb were purified from 0.8% agarose. This

fraction was ligated with the *E. coli-Mycobacterium* shuttle plasmid pMV262 (Connell et al.,

1993), linearized with *Bam*HI, and dephosphorylated. The ligation mixture was transformed into

E. coli XL10-GOLD (Stratagene, La Jolla, Calif.), and approximately 6,000 recombinants were

obtained for a theoretical representation of *P* as >99% of the *M. smegmatis* genome.

[0062]   A recombinant clone carrying the *M. smegmatis ddl* gene was identified from a

genomic library by colony hybridization using a species-specific probe. An internal fragment of

the *M. smegmatis ddl* gene was amplified by PCR using a pair of degenerate primers, DDLF and

DDLR, based on two signature peptides of bacterial Ddl enzymes (Dutka-Malen et al., 1992).

This amplified fragment was verified and radiolabeled with the Rediprime II labeling system

(Amersham Pharmacia Biotech, Piscataway, N.J.). For screening the library, about 10,000

colonies from the library pool were plated, transferred to the NYTRAN nylon membrane

(Midwest Scientific, Valley Park, Mo.), and screened with the labeled probe as described

previously (Sambrook et al., 1989). After three rounds of screening, the recombinant plasmid

pBUN172 was identified and confirmed to contain the full-length *ddl* gene. This sequence is

identical to the sequence at GenBank with accession no. AF077728 (Belanger et al., 2000) and

the sequence from the unfinished *M. smegmatis* mc^{2}155 genome (http://www.tigr.org).

[0063]   D. Drug susceptibility assays.

[0064]   MICs were determined by a microdilution method described previously (Takiff et

al., 1996) with minor modifications. Briefly, *M. smegmatis* cells were grown in M-ADC-TW to

mid-exponential phase (optical density at 600 nm, 0.6 to 1.0). Approximately 10^5 CFU in 0.1 ml

were inoculated in triplicate onto 96-well microplates containing serial twofold dilutions of

inhibitory compounds. Plates were incubated at 37°C and examined daily. The MIC was

defined as the minimal concentration of the drug or inhibitor that prevented visible bacterial

growth after 48 h. Each MIC represents the consistent result from at least three independent

cultures. Amikacin, DCS, ßCDA, ethambutol, and vancomycin (all from Sigma) were prepared

in sterile deionized water. Rifabutin (Amersham Pharmacia) was prepared in dimethyl sulfoxide

(Fisher Scientific, St. Louis, Mo.). All further dilutions of each antibiotic were prepared in

growth medium. Inhibition of colony formation by DCS was evaluated as described previously

in Caceres et al., (1997), which is incorporated herein by reference. Appropriate dilutions of

exponentially growing *M. smegmatis* cells were plated onto agar containing 0 to 1,200 µg of

DCS ml^{-1}. Colonies were counted after 5 days of incubation at 37°C. Statistical analysis was

conducted using the SAS general linear model procedure (SAS Institute, Cary, N.C.).

[0065]   E. Preparation of crude cell extracts from *M. smegmatis* strains.

[0066]   *M. smegmatis* cells were harvested at exponential phase and concentrated 50-fold

in ice-cold 50 mM Tris-HCl (pH 8.0). Cells were disrupted with a French pressure cell (Thermo

Spectronic US, Rochester, N.Y.) at 14,000 lb/in^2. The lysate was centrifuged at 4°C for 30 min

at 30,000 Xg to remove cell debris. The supernatant was subjected to ultracentrifugation at 4°C

for 4 h at 110,000 Xg to remove the membrane fraction. The recovered supernatant was

dialyzed twice against 50 mM Tris-HCl (pH 8.0) at 4°C and sterilized by filtration through 0.22-

μm-poresize filters. The protein concentration was determined by using the *DC* protein assay

(Bio-Rad) as recommended by the manufacturer.

[0067]   F. Enzyme assays.

[0068]   The crude extract was assayed for Ddl activity by a modified thin-layer

chromatography (TLC)-based method described previously (Marshall and Wright, 1998). This

procedure can quantitatively determine the amount of D-alanine:D-alanine dipeptide. Briefly,

crude extracts (5 to 30 μg of total protein) were incubated at 37°C for 4 h in a 50-μl final volume

containing 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM KCl, 50 mM unlabeled D-alanine,

6 mM ATP, 2.5 mM glutathione, and 5.0 μCi of $[1-^{14}C]$D-alanine (100 μCi/ml; ICN

Biochemicals, Inc., Costa Mesa, Calif.). For inhibition assays, DCS was added to the final

concentrations of 50, 100, 200, 400, and 800 μg ml^{-1}. Subsequently, 10 μl of the reaction

mixture was applied to cellulose-backed TLC plates (Sigma), and ascending chromatography

was developed in *n*-butanol–acetic acid–water (12:3:5) until the solvent reached the top of the

TLC plate. The plate was dried at 100°C for 5 min, and the radioactivity corresponding to the

position of the D-alanine and D-alanine: D-alanine dipeptide was measured with a

PhosphorImager. Specific activity was calculated as micromoles of D-alanine consumed per

milligram per minute. The alanine racemase activity was measured in the direction of the

conversion of L-alanine to D-alanine by a coupled spectrophotometric method (Wijsman, 1972)

with modifications (Chacon et al., 2002). Specific activity was calculated as described

previously (Caceres et al., 1997).

[0069]   G. Analysis of intracellular pools of amino acids.

[0070]   *M. smegmatis* cells were grown in minimal medium until exponential phase and

split into two subcultures, and DCS was added to a final concentration of 75 μg ml^{-1} to one of the

subcultures. After 2 h of incubation, cells were harvested at 4°C by centrifugation, washed twice

with ice-cold double-distilled water, and concentrated 50-fold. Cells were sonicated in a salt-ice-

water bath with a Vibra-Cell model VC600 sonicator (Sonic and Materials, Inc., Danbury,

Conn.) for 10 min at 80% power output and 50% duty cycle. The lysate was centrifuged at 4°C

for 30 min at 30,000 $\times g$ to remove bacterial debris. Protein was removed from the supernatant

by serial passages through YM-10 and YM-3 Centricon concentrators (Millipore Corp., Bedford,

Mass.). Determination of the abundance of individual amino acids was performed at the Amino

Acid Geochronology Laboratory of Northern Arizona University (Flagstaff, Ariz.) by a reverse-

phase high-performance liquid chromatography (HPLC) procedure described previously

(Kaufman and Manley, 1998). This procedure is able to detect nine pairs of L- and D-amino

acids in the subpicomole range. For each sample, the area of the peak representing L-glutamate

constituted 50 to 60% of the total area. Thus, the abundance of each amino acid was calculated

as the area under the corresponding peak and expressed relative to this pool.

[0071]   H. Overproduction and purification of *M. tuberculosis* Ddl enzyme

[0072]   Introduction. The development of assays to screen for and characterize novel

inhibitors of the *M. tuberculosis* Ddl enzyme requires large amounts of purified protein.

Although a preliminary biochemical characterization of inhibitors could be undertaken with

crude cell extracts from *M. tuberculosis*, a more thorough analysis of biochemical parameters

require purified products. Furthermore, large amounts of the purified protein are required for

crystallographic studies. One possibility is to purify the protein directly from *M. tuberculosis*

cell extracts. However, the purification yield is low, necessitating large cultures of a serious

human pathogen. Fortunately, recombinant DNA technology provides the means to produce

large amount of recombinant products using hosts such as *Escherichia coli*, which are safe to

handle, and easy to grow and manipulate genetically. In general, the *E. coli* system is able to

synthesize the proper mycobacterial protein; however, this is confirmed for the protein of

interest. The *M. tuberculosis* Ddl in *E. coli is designated herein as* r-Ddl. The corresponding

biochemical properties of the native *M. tuberculosis* Ddl are compared with those of r-Ddl. The

purify proteins are used to determine basic biochemical parameters including the inhibitory

parameters for the well-known inhibitor DCS and derivatives thereof.

[0073]   Likewise, the determination of biochemical parameters are performed as a means

to compare the properties of r-Ddl and the native enzyme, as well as to correlate the values

obtained in our study with those previously reported in classic studies of mycobacteria (David *et

al.*, 1969) and other microbial systems.

[0074]   Experimental design. The *M. tuberculosis ddl* gene is subcloned into an *E. coli*

overexpression vector, so that a polyhistidine-tagged Ddl protein is synthesized by the *E. coli*

recombinant clone. The fusion protein is purified from *E. coli* Ddl and other endogenous

components by one-step affinity chromatography based on the properties of the histidine tag.

Following removal of polyhistidine, polyclonal antibodies are raised against the r-Ddl and used

to purify the native *M. tuberculosis* Ddl from semi-purified extracts. Using biochemical assays,

the kinetic parameters of V_{max}, K_m for D-alanine, and K_i for DCS for both r-Ddl and native Ddl,

are measured.

[0075]   To obtain large amount of *M. tuberculosis* Ddl enzyme, the *ddl* gene is PCR

amplified based on the genome sequence and cloned into an *E. coli* expression vector to achieve

a high level of expression. Preferably, the commercially available pET system (Novagen) is

used. The advantage of this system is that the inserted gene will be under the control of the

strong T7 promoter, allowing large quantities of protein to be produced. In the pET system, the

T7 RNA polymerase gene BL21 (DE3), and is itself under the control of the lac promoter. In the

presence of the inducer IPTG, T7 RNA polymerase is expressed and will initiate transcription of

the *ddl* gene located in the plasmid. The *M. tuberculosis ddl* gene is expressed as a fusion

protein with a poly-His tag at either the N- or C- terminus, for use in purification of the resulting

protein by a single-step affinity chromatography. After optimization of protein expression, a

large-scale experiment is performed to obtain milligram levels of purified protein. The purity

and identity of the recombinant Ddl protein is verified by two-dimensional SDS-PAGE, mass

spectroscopy and N-terminal sequencing, based on the deduced protein sequence and computer-

predicted characteristics, including molecular weight and isoelectric point.

[0076]   Primers for the *M. tuberculosis ddl* gene are designed so that the 5' ends contain

the proper restriction enzyme sites to facilitate cloning. These restriction sites will be chosen

using the following criteria: the sites are not present in the *ddl* gene; and they will produce an in-

frame fusion with the His tag in the vector after cloning. Thermostable high-fidelity DNA

polymerase is used to amplify the *ddl* gene from *M. tuberculosis* genomic DNA. The resulting

PCR produce is then digested with the appropriate restriction enzymes and cloned into pET15b

following standard cloning procedures. Restriction digestion analysis and DNA sequencing of

the insertional junctions is performed to ensure the correct in-frame fusion. After verification,

the plasmid is transformed into *E. coli* BL21 (DE3), which carries the T7 RNA polymerase gene

under the control of the lac promoter. Protein expression is induced by adding 1 mM IPTG to

cultures of recombinant *E. coli* when the OD600 is between 0.5 and 1.0. Cells are harvested at

various time points after induction and whole-cell crude extracts are analyzed by SDS-PAGE to

detect the expression of the Ddl protein. Optimal conditions for protein production are obtained

from small-scale analysis of variations in initial culture density, inducer concentration, growth

temperature, agitation speed and cell density at harvest. Optimized conditions are adopted for

large-scale preparation. Cells are lysed by French Press and the resulting cell lysate passed

through a Ni affinity column. Recombinant protein with the His tag bound to the column is

eluted and the process repeated until the protein is purified close to homogeneity. The His tag in

the recombinant protein is cleaved by thrombin prior to analysis. The purity and identity of the

recombinant protein is confirmed by 2-D SDS-PAGE, mass spectrometry and N-terminal

sequencing.

[0077]   *Purification of* M. tuberculosis *native Ddl protein*. The Ddl from *E. coli* and *M.*

tuberculosis is purified to determine the identity of the recombinant and native proteins. Given

the cytoplasmic localization of the Ddl enzyme, we plan to prepare crude cell extracts of *M.*

tuberculosis H37Rv are prepared and the cytosolic fractions are separated as described above for

M. smegmatis. If necessary, further partial purification of this crude cell extract is performed by

treating the cytosolic fraction with 0.3 mg of RNase and 0.3 mg of DNase, followed by

centrifugation at 100,000 *x g* for 2 h at 4°C in a Beckman Ultracentrifuge by using a Ti 40 rotor.

To the precooled new supernatant fraction, distilled acetone is added to precipitate the

lipopolyssacharide-like components. The supernatant of the acetone precipitation is centrifuged

a 100,000 *x g* for one hour to precipitate proteins. The pellet is redissolved in buffer, and the Ddl

protein is further purified by affinity chromatography using the polyclonal antibodies raised

against the purified r-Ddl. Most of these steps are similar to those described in the classic studies

of the *M. tuberculosis* Ddl by David *et al.* (1969), except for the last affinity chromatography

step. Alternatively, the crude cell-free extract will be concentrated and loaded on the affinity

chromatography column, without additional purification steps.

[0078]   *Determination of kinetic parameters*. Using both the TLC and the P_i release

assay, the type of inhibition and the corresponding kinetic parameters are determined. The V_{max},

K_m and K_i for DCS will be evaluated by the double reciprocal plot method. Preferably, the Pi

assay is used because it is readily adaptable to a high throughput screen procedure.

EXAMPLE 2

Overexpression of the *M. smegmatis* and *M. tuberculosis ddl* genes
in recombinant *M. smegmatis*.

[0079]   The *M. smegmatis ddl* gene was isolated from a genomic library as described in

Example 1. The DNA fragment containing the *ddl* gene coding sequence, including 190 bp of

the 5' flanking region and 35 bp of the 3' flanking region, was amplified by PCR and ligated into

the *Eco*RI site of pMV262 to generate pBUN250. This plasmid was electroporated into *M.*

smegmatis mc^{2}155, yielding GPM259. Since pMV262 is present in *M. smegmatis* at 5 to 10

copies (Caceres et al., 1997), the *ddl* gene is expected to be overexpressed in the recombinant *M.*

smegmatis strain due to a gene dosage effect. This was confirmed by primer extension analysis

using total RNA isolated from mc^{2}155 and GPM259. Radiometric quantitation of the primer

extension bands confirmed that GPM259 overproduced the *ddl* transcript by 25-fold compared to

mc^{2}155. In both samples, two transcriptional start sites were detected, one immediately

upstream and another 3 nucleotides upstream of the start codon (GTG) (Fig. 1A). Further

analysis of the sequence immediately adjacent to the start codon did not reveal any significant

RNA secondary structures, excluding the possibility that the two bands detected were the result

of premature extension products. It has been found for the *Mycobacterium fortuitum blaF*

(Timm et al., 1994) and 11 streptomycete genes (Strohl, 1992) that transcription start sites are

extremely close to the start codon. This may suggest that translation initiation in both

mycobacteria and streptomycetes has less-stringent requirements for the recognition of a

ribosome-binding site.

[0080]   Following the same strategy, we constructed an *M. smegmatis* recombinant

strain, GPM198A, carrying the plasmid pBUN128A, a pMV262-based plasmid containing the

M. tuberculosis ddl gene coding sequence, including 140 bp of the 5' flanking region and 130 bp

of the 3' flanking region. Total RNA isolated from GPM198A was subjected to primer extension

analysis using oligonucleotides specific to either the *M. smegmatis* or the *M. tuberculosis ddl*

gene. The results revealed that this strain overexpressed the endogenous *M. smegmatis ddl* gene

rather than the plasmid-carried *M. tuberculosis ddl* gene. To circumvent this problem, a

different strategy was followed. Briefly, the *M. tuberculosis ddl* gene was amplified by PCR

and cloned into pMV262, yielding pBUN276. In this construct, the *M. tuberculosis ddl* gene is

fused in frame with the first six codons of the *Mycobacterium bovis* BCG *hsp60* gene and is

under the control of its promoter. Total RNA isolated from the corresponding recombinant

strain, GPM265, was subjected to primer extension analysis as described above. In this case, the

chromosomally encoded *ddl* gene in GPM265 was expressed at a level similar to the level in

wild-type mc^2155 (Fig. 1A), while the episomal *M. tuberculosis ddl* gene was expressed at an

approximately 30-fold-higher level than the chromosomally encoded *ddl* gene (Fig. 1B). The

transcriptional start site of the *M. tuberculosis ddl* gene located on pBUN276 was identified as

the G nucleotide located 183 bp upstream of the start codon of the *hsp60* gene, consistent with

previous observations (Stover et al., 1991). As expected, no *M. tuberculosis ddl* transcript was

detected from mc^2155 or GPM259 total RNA.

EXAMPLE 3

Enzymatic characterization of *M. smegmatis* strains

[0081] The overexpression of the *M. smegmatis* or *M. tuberculosis ddl* gene was further

confirmed by measuring Ddl enzyme activities. For this, Ddl-specific activities in cell crude

extracts from mc^2155, GPM259, and GPM265 were determined by the TLC-based method

described in Example 1. A typical autoradiogram of the TLC-based Ddl enzyme assays for

strain GPM259 is shown in Fig. 2A. Ddl-specific activities in crude extracts from GPM259 and

GPM265 were about 35 and 30 times greater, respectively, than that in mc^2155 extracts (Fig.

3A). These increases in Ddl activity were consistent with the primer extension results. As a

control, Alr enzyme activities were concurrently determined by a coupled spectrophotometric

method as described in Materials and Methods. As expected, cell crude extracts from mc^2155,

GPM259, and GPM265 displayed similar Alr activities (Fig. 3B), indicating that overexpression

of the *ddl* gene is specific and does not alter the expression of the *alr* gene. Furthermore, there

were no observed differences between the recombinant strains overproducing Ddl and the wild-

type strain regarding growth rate, growth saturation density, and colony morphology, indicating

that the overexpression of the *ddl* gene is not toxic to the host.

[0082] Previously, David et al. (1969) reported that DCS competitively inhibits the *M.

tuberculosis* Ddl enzyme. In this study, we performed *in vitro* inhibition assays to determine the

Ddl-specific activities of crude extracts from mc^2155, GPM259, and GPM265 in the presence of

increasing concentrations of DCS. A typical autoradiogram of the inhibition assay for the crude

extract of GPM259 is shown in Fig. 2B. The degree of inhibition for the Ddl activities in these

extracts increased proportionally to the DCS concentration (Fig. 4). No statistically significant

differences between the extracts from mc^2155 and GPM259 were observed. However, though

the inhibition of the Ddl activity in GPM265 crude extract followed a similar pattern, the

percentage of inhibition was approximately 10% higher at each concentration of DCS ($P < 0.02$).

Since the primer extension analysis indicated that more than 95% of the Ddl activity present in

the crude extract of GPM265 is from the expression of the *M. tuberculosis ddl* gene, this slight

difference may reflect a moderately higher sensitivity of the *M. tuberculosis* Ddl enzyme to

DCS inhibition. In summary, these data indicate that both *M. smegmatis* and *M. tuberculosis*

Ddl enzymes are inhibited by DCS in a concentration-dependent manner and provide further

evidence that Ddl is a target of this drug.

EXAMPLE 4

Drug susceptibilities of *M. smegmatis* strains

[0083]   To test whether Ddl overproduction confers a DCS resistance phenotype, we

evaluated the susceptibilities of *M. smegmatis* strains overproducing Ddl to DCS. The MICs of

DCS for mc^{2}155, GPM259, GPM265, and GPM14 were determined (Table 2). GPM14,

overproducing Alr, is a spontaneous DCS-resistant mutant derived from mc^{2}155 (Caceres et al.,

1997). The MICs of DCS for strains GPM259 and GPM265 (both at 150 µg ml^{-1}) were twofold

greater than that for mc^{2}155 (75 µg ml^{-1}) but were lower than that for GPM14 (300 µg ml^{-1}).

Thus, recombinant strains overproducing Ddl showed increased levels of resistance to DCS but

were not as resistant as the Alr-overproducing strain GPM14. The susceptibilities of these

strains to ßCDA, which interferes with the incorporation of D-alanine into the bacterial cell wall,

were also determined (Table 2). ßCDA, an analog of D-alanine, was reported to target bacterial

alanine racemases and transaminases (Manning et al., 1974) and showed a synergistic effect with

DCS against *M. tuberculosis* (David, 2001). In this study, only the strain overproducing Alr

showed increased resistance to ßCDA, as the MIC for GPM14, 100 µg ml^{-1}, is fourfold higher

than those for other strains with wild-type levels of Alr. These data are consistent with the mode

of action of ßCDA, which inhibits the Alr enzyme. In addition, susceptibilities to other

antimicrobial agents targeting cell wall biosynthesis (ethambutol and vancomycin), RNA

synthesis (rifabutin, a structural analog of rifampin with higher antimycobacterial activity), and

protein synthesis (amikacin) of these *M. smegmatis* strains were identical. The DCS resistance

phenotype of the Ddl-overproducing strains was further confirmed by plating bacteria on solid

agar containing various concentrations of DCS. Consistent with the MIC data, GPM259 and

GPM265 are more resistant to DCS than the wild-type mc^2155 but less resistant than the Alr-

overproducing strain GPM14 (Fig. 5). Furthermore, this type of intermediate level of resistance

to DCS is not related to Alr, since crude extracts from both GPM259 and GPM265 contained a

wild-type level of Alr activity (Fig. 3B). Therefore, overproduction of the Ddl enzymes leads to

a DCS resistance phenotype in *M. smegmatis*. However, this level of resistance may not suffice

for selection of DCS-resistant clones by following the strategy described above.

<div align="center">EXAMPLE 5</div>

Construction and characterization of an Alr-Ddl-overproducing mycobacterial strain

[0084]   It has been reported previously that a specific type of DCS-resistant *Streptococcus*

gordonii mutants displayed elevated Alr and Ddl activities and that the level of resistance to

DCS is higher than that for mutants with elevated Alr activity only (Reitz et al., 1967). To test

the effect of the overproduction of both Alr and Ddl in *M. smegmatis* on DCS susceptibility, we

introduced the multicopy plasmid pBUN250 carrying the *ddl* gene into the Alr-overproducing

strain GPM14. As expected, the resulting strain, GPM260, overproduced both Alr and Ddl as

demonstrated by enzymatic characterization (Fig. 3). In addition, the degree of inhibition of the

Ddl activities in extracts from GPM260 and its parental strain, GPM14, increased proportionally

to the DCS concentration (Fig. 4). No statistically significant differences between these extracts

<div align="center">32</div>

were observed ($P > 0.28$). Note that the overproduction of Alr in GPM14 and GPM260 does not

alter the degree of *in vitro* inhibition of the Ddl enzyme by DCS. Thus, increasing amounts of

Alr protein do not seem to affect the inhibitory effect of DCS on Ddl activity. The

susceptibilities of GPM260 to ßCDA, ethambutol, vancomycin, rifabutin, and amikacin were

identical to those of the parent strain, GPM14 (Table 2). However, GPM260, overproducing

both Alr and Ddl, was significantly more resistant to DCS than the wild-type strain or strains

overproducing only Alr or Ddl (Table 2 and Fig. 5).

[0085]   TABLE 2. MICs of DCS *M. smegmatis* strains

Strain	Phenotype		MIC^a ($\mu g\ ml^{-}1$)	
	Alr	Ddl	$ßCDA^b$	DCS^e
mc^2155	wt^e	wt	25.0	75.0
GPM2	wt	wt	25.0	75.0
GPM259	wt	Overproduced	25.0	150
GPM14	Overproduced	wt	100	300
GPM260	Overproduced	Overproduced	100	600
GPM265	wt	Overproduced*d*	25.0	150

[a] MICs were determined in M-ADC-TW media by a microdilution method as described in Materials and Methods.

[b] A chi-square test was performed for MICs obtained from at least four independent cultures. Susceptibilities to ßCDA can be divided into two groups (group I, mc^2155, GPM2, GPM259, and GPM265; group II, GPM14 and GPM260). Significant differences ($P < 0.001$) between these two groups were detected, while no significant differences (P ≥ 0.537) within each group were detected.

[c] A chi-square test was performed for MICs obtained from at least seven independent cultures. Susceptibilities to DCS can be divided into four groups (group I, mc^2155 and GPM2; group II, GPM259 and GPM265; group III, GPM14; group IV, GPM260). Significant differences between these two groups were detected (group I versus group II, $P = 0.001$; group II versus group III, P $P = 0.001$; group III versus group VI, $P = 0.008$), while no significant differences within each group were detected (group I, $P = 0.881$; group II, $P = 0.893$).

[d] More than 95% of the Ddl activity in GPM265 is due to the expression of the episomal copy of the *M. tuberculosis ddl* gene (see text).

[e]wt, wild type.

EXAMPLE 6

Analysis of the intracellular L- and D-alanine pools

[0086]   The incorporation of D-alanine into peptidoglycan requires the sequential

interconversion of L- into D-alanine followed by the formation of the D-alanine dipeptide,

reactions catalyzed by Alr and Ddl, respectively. The interplay between D-alanine biosynthesis

by Alr and its consumption by Ddl contributes to the determination of the intracellular level of

this amino acid. Inhibition of Alr alone would decrease the intracellular pool of D-alanine.

Therefore, in live bacilli exposed to sublethal concentrations of DCS (near the MIC for the wild-

type strain), if Alr is inhibited to a greater extent than Ddl, a decrease in the D-alanine pool

would be expected. To test this hypothesis, we determined the intracellular alanine pools of

mc^2155, GPM14, GPM259, and GPM260 with and without DCS treatment by a reverse-phase

HPLC. This methodology allowed detection of D- and L-alanine, L-arginine, L-

asparagine/aspartate, D- and L-glutamine/glutamate (Glx), glycine, L-isoleucine, L-leucine, D-

phenylalanine, L-serine, and D- and L-valine from samples prepared from cell extracts as

described in Example 1 (Fig. 6). It was observed that the L-Glx pool was the most abundant,

probably reflecting its crucial role in nitrogen metabolism. Moreover, this pool displayed no

significant variations upon DCS treatment ($P > 0.63$), so it was used as a standard to normalize

all other intracellular pools.

[0087]   Comparison of the amino acid pools in each strain upon DCS exposure revealed

that this treatment had a specific effect on the intracellular alanine pools (Fig. 7). In all strains

tested, DCS treatment led to a significant accumulation of L-alanine ($P < 0.03$) and a

concomitant decrease of D-alanine. The observed decrease of the D-alanine pool is statistically

significant for strains GPM259 ($P = 0.05$), GPM14 ($P = 0.01$), and GPM260 ($P = 0.01$). For

strain mc^2155, the decrease of the D-alanine pool fell below the statistically significant level ($P >$ 0.11). However, this strain displayed the maximum accumulation of L-alanine, and the L-alanine-to-D-alanine molar ratio increased 3.5-fold ($P < 0.01$).

[0088]   The intracellular pools of D- and L-alanine were also dependent on the steady-state production levels of Alr and Ddl enzymes. In cells not exposed to DCS, a twofold excess of the L-stereoisomer was observed for strains mc^2155, GMP259, and GPM 260 ($P = 0.30$) but strain GPM14 displayed almost equimolar amounts of both isomers, a result significantly different from those for the rest of the strains ($P < 0.01$). These data suggest that the overproduction of Alr favors the maintenance of a relatively abundant pool of D-alanine. Treatment with DCS led to the most significant changes, as observed by comparing effects among different strains. In the wild-type strain mc^2155, an eightfold excess of L- over D-alanine was observed. In the Alr-overproducing strain GPM14, only a three-fold excess was observed, representing a statistically significant difference from the wild-type strain ($P < 0.02$). In contrast, the Ddl-overproducing strains GPM259 and GPM260 displayed an 18- to 20-fold excess of L-alanine. This result indicated that there were no significant differences between GPM259 and GPM260 ($P > 0.80$) but that these two strains differed significantly from both the wild-type strain ($P = 0.04$) and GPM14 ($P = 0.01$). Furthermore, these changes are mostly determined by a significant reduction in the D-alanine pool compared to the values for the wild-type strain mc^2155 ($P = 0.03$) and GPM14 ($P = 0.01$). These observations suggest that, in the Ddl-overproducing strains GPM259 and GPM260, Ddl is not significantly inhibited by DCS at concentrations that inhibit Alr. Moreover, the inhibition of Alr by DCS was quite effective, since the Alr-Ddl-overproducing strain GPM260 displayed values for both the D- and L-alanine

pools similar to those displayed by strain GPM259, with wild-type Alr levels and overexpression of DdL.

EXAMPLE 7

High throughput screening of potential Ddl inhibitors

[0089]   D-alanine ligase (Ddl) activity is determined in a cell-free phosphate release assay (Piper Phosphate Assay Kit, Molecular Probes). This assay measures the amount of inorganic phosphate generated during the production of D-alanyl-D-alanine. Purified *M. tuberculosis* Ddl enzyme (Example 1H.) is used in the assay to reduce background and increase sensitivity of the assay. The assay is performed according to manufacturers instructions.

[0090]   Promising lead drug candidates are further tested *in vitro* using reference mycobacterial strains, such as *M. smegmatis* and *M. tuberculosis* Ddl overproducing strains, GPM259, GPM260, and GPM265, to evaluate the potential mycobactericidal activity of the lead compounds compared to DCS.

EXAMPLE 8

Construction of live-attenuated vaccine against Mycobacterium tuberculosis.

[0091]   Vaccine development starts with virulent *M. tuberculosis* microorganisms from which attenuated mutants are generated. These mutants preferably carry at least two attenuating deletion mutations and no antibiotic resistant markers, so as to avoid unwanted reversions or transfers of drug-resistance. Vaccine strains may include one or more additional mutations. The vaccine strain is optionally potentiated by co-administration of cytokines or by endowing the engineered strain with the capability to produce cytokines or phagosome membrane disrupters, such as lysteriolysin. Preferably, temporal expression of these genes is regulated by promoters solely active inside phagocytic cells.

[0092]   A strain of *M. tuberculosis* is constructed which is merodiploid, with an episomal copy of the *ddl* gene under the control of a promoter that is not expressed *in vivo*. The chromosomal copy of the native *ddl* gene is inactivated by means known in the art, such as point mutation or by addition, deletion, or substitution of one or more base pairs, preferably by deletion of one or more base pairs. The resulting strain is able to grow in synthetic medium but is unable to grow *in vivo*, because D-alanyl-D-alanine is not synthesized. As a result the cells undergo spontaneous lysis, releasing immunodominant B and T cell antigens. The strain may further include inactivation of the alr gene and/or may include genes for recombinant foreign antigens.

[0093]   Such strains would be useful for the generation of attenuated live-attenuated vaccine candidates against tuberculosis and other human or animal diseases caused by mycobacteria such as leprosy, Johne's disease, and possibly Crohn's disease. Because such strains are impaired in the ability to synthesize the basic building block of cell walls, peptidoglycan, the strains undergo spontaneous lysis *in vivo*. Lysis provides improved antigen delivery and containment of the vaccine strain.

DEPOSIT INFORMATION

[0094]   The recombinant mycobacterial strains GPM259, GPM260, and GPM265 have been deposited with the American Type Culture Collection (ATCC), 10801University Boulevard, Manassas, Virginia 20110-2209, USA, on _____ 200__, in accordance with the Budapest Treaty and have been accorded accession numbers _____, _____, and _____ respectively.

[0095] From the foregoing it will be seen that this invention is one well adapted to attain all ends and objectives herein-above set forth, together with the other advantages which are obvious and which are inherent to the invention.

[0096] Since many possible embodiments may be made of the invention without departing from the scope thereof, is to be understood that all matters herein set forth or shown in the accompanying drawings are to be interpreted as illustrative, and not in a limiting sense.

[0097] While specific embodiments have been shown and discussed, various modifications may of course be made, and the invention is not limited to the specific forms or arrangement of parts and steps described herein, except insofar as such limitations are included in the following claims. Further, it will be understood that certain features and sub-combinations are of utility and may be employed without reference to other features and sub-combinations. This is contemplated by and is within the scope of the claims.

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